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## HOST RECEPTORS FOR MALARIA-INFECTED ERYTHROCYTES

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**Abstract.** Mature trophozoites and schizonts of *Plasmodium falciparum* induce changes in their host erythrocyte membranes that cause infected erythrocytes to sequester by binding to capillary endothelial cells. Sequestration protects infected erythrocytes from destruction in the spleen and contributes to the pathogenesis of severe and complicated malaria. The use of in vitro parasite culture and cytoadherence assays that measure the binding of infected erythrocytes to target cells has enabled the identification of host proteins that are putative receptors to which infected erythrocytes bind. Three such receptors have been identified: the extracellular matrix protein thrombospondin, the leukocyte differentiation antigen CD36, and the intercellular adhesion molecule ICAM-1. Infected erythrocytes can bind in vitro to each of these proteins. Thrombospondin and CD36 bind to one another, but each also binds to infected erythrocytes independently. Triggering of the CD36 receptor on platelets and monocytes activates these cells in vitro, and stimulation of endothelial cells with cytokines that upregulate ICAM-1 expression can increase the binding of infected erythrocytes to endothelial cells. Studies of these and perhaps other host receptors to which infected erythrocytes bind may contribute to our understanding of pathophysiologic mechanisms in malaria.

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Mature trophozoite and schizont stages of the malaria parasite *Plasmodium falciparum* induce morphologic, functional, and antigenic changes in their host erythrocyte membranes. An important consequence of these changes is that infected erythrocytes develop the ability to sequester by binding to capillary endothelial cells.<sup>1</sup> Such sequestered infected erythrocytes do not circulate; thus they are protected from the filtering action of the spleen, which would otherwise destroy these non-deformable cells.<sup>2</sup> Sequestration may also contribute to the pathogenesis of severe and complicated malaria, either by mechanically blocking capillaries, thereby causing local hypoxia and lactic acidosis, or by activation of cells to which infected erythrocytes bind, with release of cytokines or other toxic factors.<sup>3</sup> Infected erythrocytes also bind to a variety of target cells in vitro, and cytoadherence assays have been developed that measure the binding of infected erythrocytes to these target cells.<sup>4</sup> The ability of infected erythrocytes to bind to endothelial cells and other target cells is generally correlated with the development of electron-dense knobs on the infected erythrocyte surface, although there are isolated reports of cultured parasite lines that induce knobs but do not bind to target cells in vitro<sup>5</sup> and also of cloned lines that do not induce knobs but can bind to target cells in vitro.<sup>6,7</sup>

The use of in vitro parasite culture and cytoadherence assays has enabled the identification

of several host proteins that are putative receptors to which infected erythrocytes bind. Three such receptors have been identified: the extracellular matrix protein thrombospondin (TSP),<sup>8</sup> the leukocyte differentiation antigen CD36,<sup>9,10</sup> and the intercellular adhesion molecule ICAM-1<sup>11</sup> (Table 1). Studies of these receptors may contribute to our understanding of pathophysiologic mechanisms in malaria, and may provide a useful approach to identification and characterization of the parasite-induced ligands that bind to these receptors.

### RECEPTORS

#### Thrombospondin

TSP was the first molecule identified as a receptor to which infected erythrocytes could bind.<sup>8</sup> TSP is a multi-functional glycoprotein composed of 3 identical 180 kDa subunits and appears to mediate cell-cell and cell-matrix interactions by virtue of its ability to bind to a number of other molecules including heparin, fibrinogen, fibronectin, histidine-rich glycoprotein, collagen, and plasminogen.<sup>12</sup> Roberts and coworkers<sup>8</sup> showed that TSP adsorbed to plastic specifically binds infected erythrocytes but not uninfected erythrocytes, while other adhesive proteins, such as fibronectin, laminin, von Willebrand factor, and

TABLE 1

Comparison of thrombospondin (TSP), CD36, and intercellular adhesion molecule-1 (ICAM-1) as receptors for *Plasmodium falciparum*-infected erythrocytes (IRBC)

	TSP	CD36	ICAM-1
IRBC bind to purified protein adsorbed to plastic			
Cytoadherent IRBC			
Knobby cultured lines	yes	yes	yes
<i>Aotus</i> -adapted parasites	yes	yes	ND*
Patient isolates	yes	yes	yes
Non-cytoadherent IRBC			
Ring-infected erythrocytes	no	no	no
Knobless cultures lines (mature forms)	no	no	no
Trypsin-treated IRBC	no	no	ND
Specific, saturable binding of protein to IRBC	ND	yes	ND
IRBC bind to transfected cells expressing protein	ND	yes	yes
Purified protein inhibits binding of IRBC			
To homologous protein adsorbed to plastic	yes	yes	ND
To melanoma cells in vitro	yes†	yes	ND
To endothelial cells in vitro	ND	yes	ND
To rat venular endothelium ex vivo	yes	ND	ND
Antibodies to protein inhibit binding of IRBC			
To homologous protein adsorbed to plastic	yes	yes	yes
To melanoma cells in vitro	yes‡	yes	ND
To endothelial cells in vitro	yes	yes	yes
To rat venular endothelium ex vivo	yes	ND	ND
Correlation of cytoadherence and expression of receptor on melanoma cells	no	yes	no

\* ND = not determined.

† The ability of thrombospondin to inhibit binding of infected erythrocytes to melanoma cells may depend on the conformational integrity of the protein, since we have been unable to inhibit binding of infected erythrocytes to melanoma cells with several different lots of thrombospondin obtained from D. D. Roberts, National Institutes of Health, Bethesda, MD (data not shown).

‡ Results vary with different antisera.

vitronectin, do not bind infected erythrocytes. The ability of infected erythrocytes to bind to target cells in vitro varies among different cultured isolates of *P. falciparum*, and there is a good correlation between the ability of infected erythrocytes to bind to melanoma cells and their ability to bind to TSP for both laboratory-adapted strains<sup>8</sup> and wild isolates.<sup>13</sup> Only erythrocytes infected with mature trophozoite and schizont stages of the parasite bind to TSP,<sup>13</sup> similar to the stage-specificity of infected erythrocyte binding to endothelial cells and melanoma cells.<sup>14,15</sup> In addition, the ligand that binds to TSP and the ligand that binds to melanoma cells are both sensitive to cleavage from the surface of infected erythrocytes by trypsin.<sup>2,13</sup>

Further evidence suggesting that TSP is a receptor on melanoma cells to which infected erythrocytes bind was obtained by showing that soluble TSP, rabbit TSP antibody, and a mouse monoclonal TSP antibody each inhibits binding of infected erythrocytes to melanoma cells.<sup>8</sup> Each reagent is more potent at inhibiting binding of

infected erythrocytes to TSP on plastic than to melanoma cells. The ability of TSP antibodies to inhibit binding of infected erythrocytes to melanoma cells appears to depend on the specificity of the antiserum or the techniques of performing the assay, since several investigators have not been able to inhibit binding of infected erythrocytes to melanoma cells with a number of different rabbit TSP antisera,<sup>10,16,17</sup> and others have found only limited inhibition.<sup>18</sup>

Additional data supporting a role for TSP in cytoadherence was obtained by Rock and co-workers using a rat ex vivo mesoecum model.<sup>19</sup> In this model, the mesoecal vasculature is perfused with lactated Ringer's solution under constant venous pressure. After infusing a bolus of infected or uninfected erythrocytes, obstruction of blood flow is indicated by an increase in the peripheral resistance, which is calculated from the arterial pressure and venous outflow rate, and by a prolongation in the pressure flow recovery time, which is the time required for arterial pressure and venous flow to return to normal after

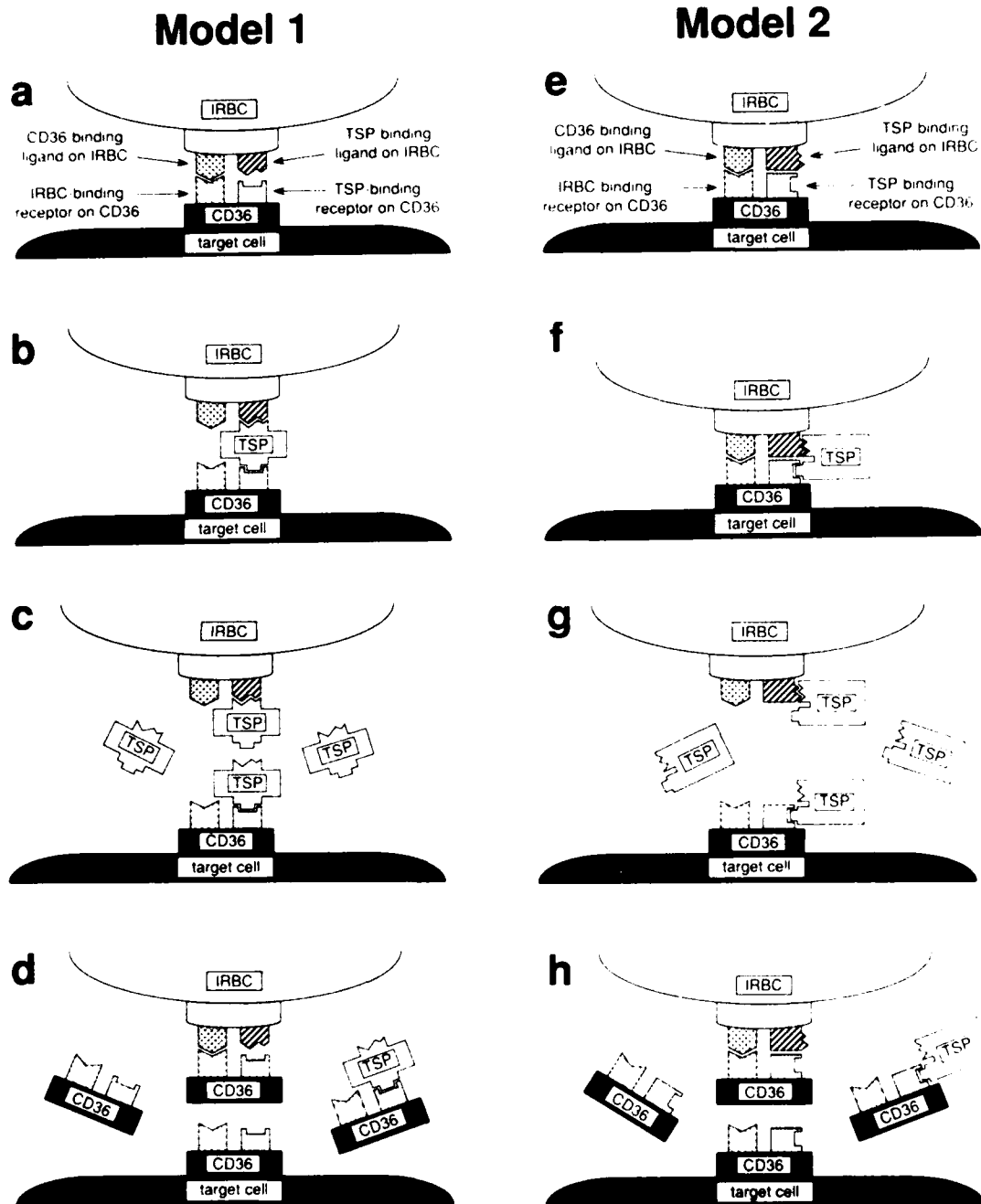


FIGURE 1. Theoretical models of the interaction of malaria-infected erythrocytes (IRBC), thrombospondin (TSP), and CD36.

infusing a bolus of cells. In addition, the flow of cells is monitored by direct microscopy and simultaneous video recording of the microcirculation. Infusion of control uninfected erythro-

cytes causes little change in peripheral resistance, and arterial pressure and venous flow generally return to normal within 2 min after the infusion. Infusion of infected erythrocytes causes a marked

increase in peripheral resistance, and arterial pressure and venous flow generally do not return to normal after the infusion. By microscopy, these changes are associated with adherence of infected erythrocytes to venular endothelium and blockage of some vessels. Pre-incubation of infected red cells with soluble TSP inhibits binding of infected erythrocytes to venular endothelium and reduces the changes in peripheral resistance and pressure flow recovery time towards normal. Inhibition is observed at TSP concentrations of 120–300  $\mu\text{g/ml}$ , which is  $\sim 10,000$ -fold above the normal plasma concentration of 20  $\text{ng/ml}$ ,<sup>20</sup> but is not observed at TSP concentrations of 30  $\mu\text{g/ml}$ , which is 1,000-fold above the normal plasma concentration. Similarly high concentrations of TSP are required for inhibition of infected erythrocyte binding to melanoma cells.<sup>8</sup> In the rat mesocecal vasculature model, cytoadherence can also be inhibited by pre-incubation of infected red cells with human immune serum, or by infusion of the microcirculatory preparation with rabbit TSP antibodies before infusion of infected erythrocytes.

There have been a few results, however, that do not support the hypothesis that TSP alone is the receptor to which infected erythrocytes bind. Panton and coworkers evaluated a number of melanoma cell lines and correlated their ability to bind infected erythrocytes with their ability to synthesize and express TSP.<sup>21</sup> Only 2 of 7 cell lines were able to bind infected erythrocytes, but all 7 cell lines synthesized TSP, as determined by immunofluorescence of fixed cells with TSP antibodies. None of the cell lines expressed TSP on the cell surface, as determined by immunofluorescence of living cells with TSP antibodies. Sherwood and coworkers used sensitive radioimmunoassay techniques to examine some of these melanoma cell lines in more detail and found no major differences between binding and non-binding melanoma cells in the amount of TSP they secreted into the culture medium, the amount of TSP they expressed on their surface, or the number of surface receptors capable of binding added TSP.<sup>22</sup>

### CD36

In addition to TSP, an 88 kDa glycoprotein has been identified as a receptor for malaria-infected erythrocytes. This glycoprotein is present on the surface of a number of cells to which

infected erythrocytes bind *in vitro*, including endothelial cells, monocytes, platelets, and certain melanoma cell lines.<sup>23,24</sup> On monocytes, this protein is known as CD36. The CD nomenclature is used to name leukocyte differentiation antigens that are recognized by clusters of monoclonal antibodies (Mabs), and are thus referred to as cluster determinants, or CDs. OKM5 is one of the Mabs that recognizes CD36, and the protein is sometimes referred to as the OKM5 antigen. On platelets, this protein is known as glycoprotein IV<sup>25</sup> or glycoprotein IIb,<sup>26</sup> and on melanoma cells has been called GP88.<sup>10</sup> We use the term CD36 to refer to this protein from any source.

The first evidence supporting a role for CD36 in cytoadherence was the observation by Barnwell, Ockenhouse, and Knowles that Mab OKM5 inhibits the binding of malaria-infected erythrocytes to monocytes, melanoma cells, and endothelial cells.<sup>23</sup> In addition, the surface expression of CD36 on target cells correlates with the ability of these cells to bind infected erythrocytes. Only 2 of 7 melanoma cell lines tested by Panton and coworkers were able to synthesize and express CD36 on their surface, and these were the only 2 cell lines that were able to bind infected erythrocytes.<sup>21</sup> We examined the myelomonocytic cell line U937 and showed that only about 8% of unstimulated cells expressed CD36 and were able to bind infected erythrocytes. However, after stimulation with phorbol myristate acetate to induce further differentiation along the monocyte pathway,  $\sim 80\%$  of cells expressed CD36 and were able to bind infected erythrocytes.<sup>16</sup>

We obtained direct evidence that CD36 is a receptor to which infected erythrocytes bind using CD36 purified from platelets.<sup>9</sup> Similar results were obtained by Barnwell and coworkers using CD36 purified from melanoma cells or platelets.<sup>10</sup> Knobby infected erythrocytes bind specifically to CD36 adsorbed on plastic,<sup>9,10</sup> and <sup>125</sup>I-labeled CD36 shows specific saturable binding to knobby infected erythrocytes but not to uninfected erythrocytes or to knobless infected erythrocytes that do not bind to endothelial cells or melanoma cells *in vitro*.<sup>9</sup> Binding of infected erythrocytes to CD36 is stage-specific, limited to erythrocytes infected with mature trophozoites and schizonts, and requires a trypsin-sensitive ligand on the infected erythrocyte surface.<sup>9,10</sup> Incubation of infected erythrocytes with soluble

CD36 inhibits their ability to bind to melanoma cells, endothelial cells, monocytes, and platelets *in vitro*.<sup>9,10,24</sup> Infected erythrocytes can also bind to transfected COS cells expressing CD36 on their surface.<sup>18</sup>

A preliminary report had suggested that CD36 might not be present on normal cerebral capillary endothelium, since Mab OKM5 did not react with these cells in immunocytochemical staining.<sup>27</sup> More recent studies using other CD36 Mabs have confirmed the presence of CD36 on cerebral capillary endothelium of both normal individuals and patients with cerebral malaria.<sup>10,28</sup>

Triggering of the CD36 receptor on platelets and monocytes by binding of infected erythrocytes or CD36 Mabs results in activation of these cells *in vitro*.<sup>24</sup> In platelets, this activation results in platelet aggregation and granule release. In monocytes, this activation causes stimulation of the respiratory burst and production of reactive oxygen intermediates,<sup>24</sup> which are thought to play a role in the pathophysiology of malaria.<sup>29</sup> CD36-mediated activation of both monocytes and platelets requires appropriate intracellular transmembrane signaling and is inhibited by calcium antagonists or inhibitors of protein kinase C or guanine nucleotide binding proteins.<sup>24</sup> These data indicate that, at least on platelets and monocytes, CD36 is a receptor in the pharmacologic sense, i.e., a membrane protein that responds to an agonist by transmembrane signaling. If CD36 on endothelial cells also behaves as a pharmacologic receptor, interaction of infected erythrocytes with endothelial cells may have important consequences on endothelial function.

#### *Interactions of CD36 and TSP*

Several lines of evidence indicate that CD36 and TSP bind to one another. Asch and co-workers showed that Mab OKM5 inhibits binding of iodinated TSP to platelets, fibrosarcoma cells, and C32 melanoma cells.<sup>30</sup> These authors and McGregor and coworkers<sup>26</sup> also showed that purified CD36 binds to immobilized TSP and purified TSP binds to immobilized CD36, suggesting that CD36 is the TSP membrane receptor.<sup>30</sup> However, TSP contains an Arg-Gly-Asp (RGD) sequence that appears to mediate its binding to integrin-like receptors on a number of cell types, since hexapeptides containing RGD inhibit the binding of TSP to these cells.<sup>31,32</sup> Additional data regarding the interaction of TSP

with CD36 and other platelet membrane components has been obtained by precipitating <sup>125</sup>I surface-labeled platelet membrane extracts with Mabs specific for TSP or fibrinogen.<sup>33</sup> When platelets are activated in calcium-replete medium, TSP forms a complex with fibrinogen and at least 5 platelet surface molecules, including CD36 and the platelet integrin receptor GPIIb/IIIa. All of these molecules are co-precipitated from platelet membrane extracts by Mabs specific for TSP or fibrinogen. When platelets are activated in the absence of calcium, however, TSP binds preferentially to CD36; CD36 is the predominant surface-labeled protein co-precipitated from platelet membrane extracts by a Mab specific for TSP, and no labeled proteins are co-precipitated by a Mab specific for fibrinogen.<sup>33</sup>

Because TSP binds to both CD36 and to infected erythrocytes, it was possible that TSP might mediate the binding of infected erythrocytes to CD36. However, Barnwell and coworkers<sup>10</sup> have shown that infected erythrocytes can bind to CD36 independent of TSP. Binding of infected erythrocytes to CD36 is independent of calcium and occurs in the presence of EDTA, while binding of TSP to infected erythrocytes occurs only in the presence of calcium. In addition, infected erythrocytes cultured in serum-free medium that contains no detectable TSP, or in medium containing serum from which TSP has been depleted by immunoadsorption, bind to purified CD36 equally well as infected erythrocytes cultured in medium containing TSP.<sup>10</sup>

To interpret these findings, Barnwell and his colleagues have proposed a model in which TSP has 1 domain that mimics the infected erythrocyte ligand that binds to CD36 and another that mimics the CD36 receptor that binds to the infected erythrocyte ligand.<sup>10</sup> We would propose a slightly different model for the interaction of CD36, TSP, and infected erythrocytes (Fig. 1). In this model, there are separate ligands on the infected erythrocytes that bind independently to TSP and to CD36. The evidence for this includes the observation that soluble TSP and CD36 inhibit binding of infected erythrocytes to the homologous but not the heterologous protein immobilized on plastic (data not shown) and the observation that treatment of infected erythrocytes with certain enzymes can abrogate binding of infected erythrocytes to CD36 but not to TSP (data not shown). The 2 infected erythrocyte ligands could either be separate domains on 1 mol-

TABLE 2

Binding of infected erythrocytes isolated from Thai patients with uncomplicated or severe malaria to ICAM-1 and CD36\*

Type of malaria	Infected erythrocytes per mm <sup>2</sup> bound	
	ICAM-1	CD36
Uncomplicated (n = 9)	109 ± 138† (4–538)‡	499 ± 254 (160–934)
Severe (n = 9)§	155 ± 145 (380–1,132)	711 ± 272 (34–488)

\* Parasite isolates were obtained in collaboration with Kyle Webster, May Ho, and Nick White in Thailand; purified CD36 was obtained from Narendra Tandon and Graham Jamieson at the American Red Cross, Rockville, MD; purified ICAM-1 was obtained from Gijs van Seventer and Stephen Shaw at the National Institutes of Health, Bethesda, MD.

† Mean ± SD.

‡ Range.

§ Includes 5 patients with unrousable coma, 2 with hepatic failure and renal failure, and 1 each with renal failure or pulmonary edema and stupor.

ecule, or they could be on 2 different, but probably interacting, molecules. There also appear to be 2 separate domains on CD36 that bind to TSP and to infected erythrocytes, since binding of infected erythrocytes to CD36 on monocytes and platelets causes transmembrane signaling and activation of these cells, but binding of TSP does not. The binding of infected erythrocytes to CD36 on target cells via the CD36-binding ligand and via TSP might be mutually exclusive events (Fig. 1a, b). Alternatively, simultaneous binding of TSP to infected erythrocytes and to CD36 on host cells might occur, thus enhancing the stability of the infected erythrocyte-host cell interaction (Fig. 1f). In either case, a vast excess of soluble TSP would inhibit the interaction of infected erythrocytes with host cells because all the TSP-binding ligands on infected erythrocytes would be occupied by TSP that was not bound to CD36, and all the TSP-binding receptors on CD36 would be occupied by TSP that was not bound to infected erythrocytes, with resultant steric hindrance of the interaction of the CD36-binding ligand on infected erythrocytes with its receptor on CD36 (Fig. 1c, f). An excess of CD36 (Fig. 1d, h) or antibodies to CD36 or TSP would also block the interaction of infected erythrocytes with target cells by steric hindrance.

### ICAM-1

In addition to TSP and CD36, ICAM-1 has also been shown to be a receptor to which *P. falciparum*-infected erythrocytes bind.<sup>11</sup> ICAM-1 is a cell surface glycoprotein that mediates cell-

cell interactions by binding to the leukocyte function antigen LFA-1. Berendt and coworkers<sup>11</sup> studied 2 laboratory strains of *P. falciparum*, ITO4 and FCR3, which differ in their cytoadherence properties. Erythrocytes infected with FCR3 parasites bind to C32 melanoma cells but not to primary human umbilical vein endothelial cells, while erythrocytes infected with ITO4 parasites, which were selected for endothelial cell binding, bind to both melanoma cells and endothelial cells. When COS cells transfected with either CD36 or ICAM-1 genes are used as target cells to which infected erythrocytes can bind, erythrocytes infected with either FCR3 or ITO4 parasites bind to transfected COS cells that express CD36 on their surface, while erythrocytes infected with ITO4 parasites, but not FCR3 parasites, bind to transfected COS cells that express ICAM-1 on their surface. Thus erythrocytes infected with ITO4 parasites express ligands that bind to both CD36 and ICAM-1, but erythrocytes infected with FCR3 parasites express only the ligand that binds to CD36. A Mab to CD36 blocks the binding of infected erythrocytes to the CD36 transfectants and to melanoma cells, but has little effect on the binding of ITO4-infected erythrocytes to endothelial cells. In contrast, a Mab to ICAM-1 blocks the binding of ITO4-infected erythrocytes to the ICAM-1 transfectants and to endothelial cells, but has little effect on the binding to melanoma cells. These data suggest that binding of ITO4-infected erythrocytes to melanoma cells is mediated primarily by CD36, while binding of ITO4-infected erythrocytes to cultured endothelial cells is mediated primarily by ICAM-1.

ICAM-1 expression can be up-regulated on endothelial cells in response to interleukin-1, tumor necrosis factor (TNF), and bacterial endotoxin, and these stimuli also increase the ability of cultured endothelial cells to bind ITO4-infected erythrocytes.<sup>11</sup> Because serum TNF levels are increased in acute malaria and correlate with the severity of disease,<sup>34</sup> this raises the intriguing possibility that cerebral malaria and other manifestations of severe disease may occur when parasites that express a ligand that can bind to ICAM-1 infect an individual who can express high levels of ICAM-1 on his endothelial cells.<sup>11</sup>

In order to gain data that might support this possibility, we evaluated the ability of parasite isolates from Thai patients with uncomplicated or severe malaria to bind to purified proteins and

to melanoma cells in vitro. The mean number of infected erythrocytes per mm<sup>2</sup> bound to ICAM-1 was not significantly different for isolates from 9 patients with severe malaria and 9 patients with uncomplicated malaria. There was also no significant difference in the mean number of infected erythrocytes per mm<sup>2</sup> bound to CD36 for isolates from patients with severe malaria and patients with uncomplicated malaria (Table 2).

#### Multiple host receptors

Why should there be at least 3 distinct receptors to which infected erythrocytes bind? Malaria parasites have developed many variable or redundant mechanisms to survive in the hostile environment of a host that is able to mount an immune response. For example, many malaria antigens show considerable variability in both B cell and T cell epitopes. In addition, malaria merozoites require specific receptor-ligand interactions in order to invade host erythrocytes, and studies by Hadley and coworkers<sup>35</sup> have demonstrated the existence of alternative merozoite invasion pathways. Most isolates of *P. falciparum* utilize sialic acid on erythrocyte glycophorin as a receptor to which merozoites bind, and treatment of erythrocytes with neuraminidase, which removes surface glycophorin, almost completely prevents merozoite invasion in vitro.<sup>35</sup> Merozoites of some isolates, however, can invade glycophorin-deficient or neuraminidase-treated erythrocytes with ~50% efficiency. The ability of these merozoites to invade erythrocytes can be almost completely prevented by treatment of erythrocytes with trypsin. It has been postulated that this sialic acid-independent, trypsin-sensitive, alternative invasion pathway may allow parasite strains to survive in a host that has developed immune mechanisms that inhibit the usual, sialic acid-dependent invasion pathway.<sup>35</sup> Perhaps binding of infected erythrocytes to TSP, CD36, and ICAM-1 similarly represents alternative mechanisms for survival of parasite strains in a semi-immune host. This may be an especially important consideration if attempts to develop a subunit vaccine based on the infected erythrocyte ligands that recognize host endothelial receptors are successful. There may also be other receptors not yet identified that bind to ligands on infected erythrocytes.

#### Identification of infected erythrocyte ligands

In addition to potential insights into the pathogenesis of malaria, a major reason for studying receptors that bind infected erythrocytes is to try and identify the complementary parasite-induced ligands on the infected erythrocyte surface.<sup>36</sup> Because sequestration appears to be critically important for continuation of the parasite's life cycle,<sup>2</sup> and because immune serum can inhibit and reverse cytoadherence<sup>37</sup> and sequestration,<sup>2</sup> immunization with relevant epitopes from cytoadherence ligands should be beneficial to the immunized host and detrimental to the parasite. Several groups are using the receptor proteins identified so far to try and identify the complementary ligands, either by immunochemical techniques or by cloning the genes that encode the ligands.

#### CONCLUSIONS

Cytoadherence is more complex than some might have predicted. At least 3 host receptors have been identified that bind to ligands on the surface of malaria-infected erythrocytes, and at least 2 of these receptors interact with one another. All of the receptor studies that have been performed thus far have involved in vitro or ex vivo models, and it is not certain any are directly relevant to the in vivo situation. The search for answers to these problems will undoubtedly lead to exciting solutions, but also to additional unanswered questions.

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